International Journal of Chemistry Studies
ISSN: 2581-348X
Impact Factor: RJIF 5.44
www.chemistryjournal.in
Volume 2; Issue 1; January 2018; Page No. 20-25

NMR determination of bioactive compound Isolated from the Aerial parts of *Vernonia Pauciflora* (Willd.) Less

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Abstract
Vernonia pauciflora is a plant found in the North central Nigeria and is used in the traditional treatment of many diseases including pneumonia, tuberculosis, skin infection, meningitis, whooping cough, typhoid fever, headache, and diarrhoea. The result of the zones of inhibition of growth of the microorganisms ranged from 17-20 mm for n-hexane extract, 22-28 mm for ethyl acetate and 20-25 mm for the methanol extract. The Minimum Inhibition Concentration (MIC) result for n-hexane, ethyl acetate and methanol extracts ranged from 7.5-30 mg/mL while the Minimum Bactericidal Concentration/Minimum Fungicidal Concentration (MBC/MFC) result for the extracts ranged from 15-60 mg/mL. Extensive chromatographic separation of the ethyl acetate extract led to the isolation of compound ADT1. The structure of the compound was established as 3-oxolanyl acetate using spectral analysis including 1D and 2D NMR. The antimicrobial activity of the isolated compound as indicated by the zone of inhibition ranged from 22-29 mm against *Methicilin Resistant Staphylococcus aureus* (*MRSA), Vancomycin Resistant enterococci (VRE), Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Candida albicans*, and *Candida krusei*.

Keywords: *Vernomia pauciflora* (Willd.) Less, NMR spectral analysis, antimicrobial activity

1. Introduction

1.1 *Vernonia pauciflora*

Vernonia is a genus of about 1000 species in the family Asteraceae. They are common in most West African and Central African countries. Some species are known as ironweed. *Vernonia* species are usually annual herbs that grow up to 3-5 m, mostly they have much smaller stem with ribs, finely to coarsely hairy, sometimes has branches near the top. *Vernonia* species are found mostly in semi-arid tropics where it is found in dry bush land, although *Vernonia pauciflora* is widely distributed in wet and dry forest environment (Jeffrey, 1998) [5].

2. General medicinal properties of *Vernonia* Species

The genus *Vernonia* is enriched with pharmacological properties. Various species of genus *Vernonia* are used for the treatment of schistosomiasis, amoebic dysentery, gastrointestinal problems, malaria, venereal diseases, wounds, hepatitis, diabetes, intestinal colic, fever, stomachache, toothache, cough, nasal and bronchial pain (Erasto *et al.* 2006) [3].

Fig 1: *Vernonia pauciflora (Willd.)Less* in its Natural Habita

2.1 Pharmacological/biological studies of some *Vernonia* species and isolated compounds

Vernodalol isolated from *V. amygdalina* exhibited significant activity against some microorganisms. It showed moderate inhibition against *Aspergillus flavus*, *Penicillium notatum* and *Aspergillus niger* with LC50 values of 0.3, 0.4 and 0.5 mg/ml, respectively (Erasto *et al.* 2006) [3]. Vernodalin and vernolepin isolated from the same plant are used as antibacterial and antifungal agent (Erasto *et al.* 2006) [3]. Zaluzanin D isolated from *V. arborea* showed 100 % inhibition in mycelial growth of *Rhizoctonia solani*. Vernolepin isolated from methanolic extract of *V. cinerea* demonstrated potent cytotoxicity against human and Hela tumor cell lines (Kumari *et al.* 2003) [6]. Vernodalin isolated from ethyl acetate extract of *V. bockiana* showed strong cytotoxicity against mouse lymphoid tumor cell line with IC50 values of 0.73 µM (Huo *et al.* 2008 [4].

3. Plant Material

3.1 Collection, identification and preparation of plant material

The plant material was collected from Makurdi, Benue State, Nigeria in the month of November, 2013. It was identified and authenticated at the Herbarium unit by Mallam Musa Mohammed of Biological Sciences Department, Ahmadu Bello University, Zaria, Nigeria; where it was assigned the specimen voucher number 1403.The aerial parts of the plant (stem, leaves and flowers) were air-dried for 21 days and pulverizedn to coarse powder.

3.2 Extraction of Plant Material

The powdered plant material (800 g) was carefully weighed and extracted successively with n-hexane, ethyl acetate and methanol using cold maceration till exhaustion. The various extracts were then carefully concentrated in *vacuo* at 40 ºC using a rotary evaporator under controlled temperature and reduced pressure and subjected to air drying to give dried crude extracts.

3.3 Antimicrobial Screening

3.3.1 Test Organisms

The microorganisms tested were: *Methicilin Resistant Staphylococcus aureus (MRSA), Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Neisseria gonorrhoae, Salmonella typhi, Vancomycin Resistant enterococci (VRE), Streptococcus pyogenes, Candida albicans, and Candida krusei.* They were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika, Zaria. All the isolates were checked for purity and maintained in nutrient agar slant.

3.3.2 Culture Media

The culture media used were Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB). All media were prepared according to manufacturer's instruction.

3.3.3 Preparation of Inocula of Test Organisms

The McFarland turbidity standard scale 1 was used to standardize the organisms. The scale was prepared by adding 9.9 mL of 1 % sulphuric acid to 0.1 mL of 1 % Barium Chloride ($BaCl_2$).The volumes of the prepared stock solution were mixed according to the McFarland nephelometer standard. Suspensions of the organisms were in sterile distilled water and compared with the McFarland turbidity standard, until the opacity matched with the scale number 1, which corresponds to $3.6x10^6$ bacterial densities (McFarland, 1907).

3.3.4 Sensitivity Test

The agar-in-well diffusion method was used (Nostro, 2000) [8]. The antimicrobial activities of the n-hexane, ethyl acetate and methanol extracts of the aerial parts of *Vernonia pauciflora* were determined using stock concentration of 10 mg/mL. The standardized inocula of the isolates were uniformly streaked unto freshly prepared Mueller Hinton agar plates with the aid of a sterile swab stick. Using a sterile cork borer (6 mm in diameter), 5 appropriately labeled wells were punched into each agar plate. Aliquot of 0.2 mL of the appropriate extract concentration was placed in each well and then allowed to diffuse into the agar. An extra plate was streaked with the isolate and ciprofloxacin (5 µ/disc) was placed on it. The plates were incubated at 37 ºC for 24 h. While for the fungi, Sabouraud dextrose broth was used and the incubation period was 30 ºC and 48 h. The antimicrobial activities were expressed as diameter (mm) of inhibition zones produced by the plant extracts.

3.3.5 Determination of Minimum Inhibition Concentration (MIC)

The minimum inhibition concentrations of the extracts were carried out on the test microbes using the broth dilution method as outlined by the Clinical and Laboratory Standards Institute (CLSI, 2014) [2]. Mueller Hinton broth was prepared; 10 mL was dispensed into tubes and was sterilized at 121 ^{0}C for 15 minutes and allowed to cool. The McFarland turbidity standard scale 0.5 was prepared to give turbidity solution. Normal saline was prepared, 10 mL was dispense into sterile test tube and the test microbes was inoculated and incubated at 37 ^{0}C for 24 h. Dilution of the test microbes was done in the normal saline until the turbidity marched that of the Mc-Farland turbidity scale by visual comparison at this point the test microbe has a concentration of about $1.5x10^8$ cfu/mL. Two-fold serial dilution of the extracts in the sterilized broth was made to obtain the concentrations of 60 mg/mL, 30 mg/mL, 15 mg/mL, 7.5 mg/mL, and 3.25 mg/mL. The initial concentration was obtained by dissolving 0.6 g of the extract in 10 mL of the sterile broth. Having obtained the different concentrations of the extracts in the sterile broth, 0.1 mL of the standard inoculum of the test microbe in the normal saline was then inoculated into the different concentrations. Incubation was made at 37 ^{0}C for 24 h, after which each test tube of the broth was observed for turbidity (growth). The lowest concentration of the extract in which the broth shows no turbidity was recorded as the Minimum Inhibition Concentration (MIC).

3.3.6 Determination of Minimum Bactericidal Concentration/Minimum Fungicidal Concentration (MBC/MFC)

The minimum bactericidal concentration of the extracts was determined as outlined by the CLSI on the nutrient agar plates.

Minimum bactericidal concentrations were determined by assaying the test tube contents of the MIC determinations. A loopful of the content of each tube was inoculated by streaking on a solidified nutrient agar plate and then incubated at 37 ^{o}C for 24 h for bacterial and 30 ^{o}C for 48 h for fungi. After which it was observed for microbial growth. The lowest concentration of the subculture with no growth was considered as minimum bactericidal concentration/ minimum fungicidal concentration.

3.4 Chromatographic Procedure

3.4.1 Thin Layer Chromatography, TLC

Thin layer chromatography was carried out on TLC aluminium sheet silica gel 60 PF_{254} pre coated with layer thickness of 0.2 mm.

3.5 Spectroscopic Analysis

Different spectroscopic methods were used to elucidate the structure of isolated compound ADT1. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded using $CDCl_3$ as solvent on Bruker DRX 600MHz NMR spectrometer at the School of Chemistry and Physics, University of Kwazulu-Natal, South Africa.

3.6 Sensitivity Test of ADT1

The sensitivity test of compound ADT1 was carried out using the same method used for the crude extracts and with same test organisms.

3.7 Melting Point Result

The melting point range of compound ADT1 was found to be 130-133^{0}C

4. Results

4.1 Antimicrobial Activity

The results of the antimicrobial susceptibility tests, expressed in terms of the diameter of inhibition zones of the test organisms are shown in Table 1. The results of the zone of inhibition showed that the plant extracts had remarkable activity against the tested microorganisms with inhibition zones ranging from17-28 mm.

The minimum inhibition concentration (MIC) of the test organisms are shown in Table 2. The MIC values ranged from 7.5-30 mg/mL.

The Minimum bactericidal concentration/ minimum fungicidal concentration (MBC/MFC) of the test organisms are shown in Table 3. The MBC/MFC values ranged from 15-60 mg/mL for all the plant extracts tested against the microorganisms.

Table 1: Determination of Zone of Inhibition (mm) of the extracts and standard drugs

Test organism	EtOAc	MeOH	n-Hex	Ciprofloxacin 5 µg/disc	Fluconazole 5 µg/disc
MRSA	27	25	20	32	-
S. aureus	24	22	18	31	-
S. pyogenes	0	0	0	35	-
S. pneumoniae	28	23	20	33	-
E. coli	24	20	18	37	-
N. gonorrhoae	25	21	17	0	-
S. typhi	22	20	18	42	-
S. dysenteriae	0	0	0	0	-
C. albicans	24	20	17	-	35
C. krusei	23	20	18	-	37

Key: n-hex=Hexane, EtOAc =Ethyl acetate, MeOH=Methanol

Table 2: Results of Minimum Inhibition Concentration (MIC) Determination (mg/mL)

Test organism	EtOAc	MeOH	n-Hex
MRSA	7.5	15	15
S. aureus	15	15	30
S. pneumoniae	7.5	15	15
E. coli	15	15	30
N. gonorrhoae	15	15	30
S. typhi	15	15	30
C. albicans	15	15	30
C. krusei	15	15	30

Key: n-hex=Hexane, EtOAc =Ethyl acetate, MeOH=Methanol

Table 3: Determination of minimum bactericidal concentration/minimum fungicidal concentration, MBC/MFC (mg/mL)

Test organism	EtOAc	MeOH	n-Hex
MRSA	30	30	60
S. aureus	30	60	60
S. pneumoniae	15	30	60
E. coli	15	30	60
N. gonorrhoae	30	60	60
S. typhi	60	60	60
C. albicans	60	60	60
C. krusei	60	60	60

Key: n-hex=Hexane, EtOAc =Ethyl acetate, MeOH=Methanol

4.2 Chromatographic Separation

4.2.1 Thin layer chromatography of ethyl acetate extract

Thin layer chromatography (TLC) was carried out on the ethyl acetate extract, and the results are shown in Table 4. Three prominent spots were observed (Hex: EA 7:3) on the TLC plate after spraying with 10 % H_2SO_4, followed by heating at 110 ºC for 5-10 minutes. The retention factor (R_f) of the three spots are 0.66, 0.78 and 0.96.

Table 4: Results of TLC of Ethyl Acetate Extracts Determination

Solvent system	No of spot	R_f value	Colour in 10 % H_2SO_4
Hex: EA(7:3)	3	0.66	Yellow
		0.78	Orange
		0.96	Purple
Hex: EA(1:1)	2	0.51	Orange
		0.53	Yellow
Hex: EA(9:1)	2	0.36	Purple
		0.48	Orange

4.3 Column chromatography of ethyl acetate fraction

Column chromatography of ethyl acetate extract (8 g) was carried out and the results shown in Table 4.7. The column was eluted continuously using neat n-hexane, followed by different n-hexane: ethyl acetate solvent systems, then methanol.

4.4 Antimicrobial Activity of 3-oxolanyl acetate

The results of the antimicrobial susceptibility tests, expressed in terms of diameter of zones of inhibition are shown in Table 4.11. The test showed the activities against *MRSA, VRE, S. aureus, S. pyogenes, E. coli, C. albican* and *C. krusei* using agar well diffusion method.

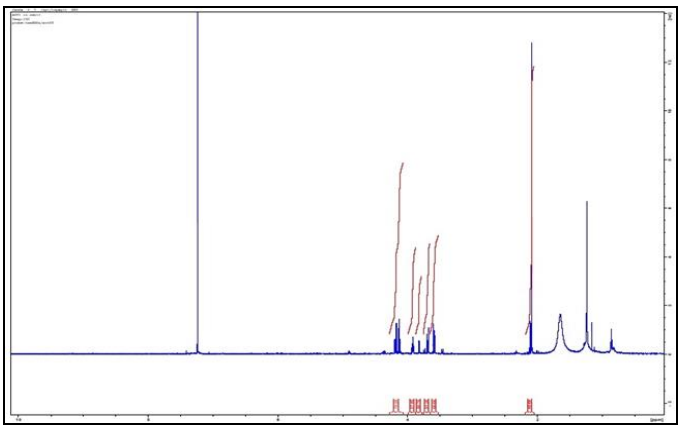

Fig 2: ^{1}HNMR of ADT1

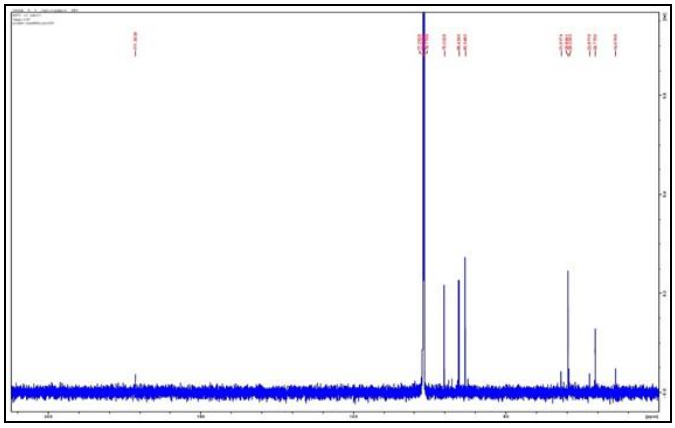

Fig 3: ^{13}CNMR of ADT1

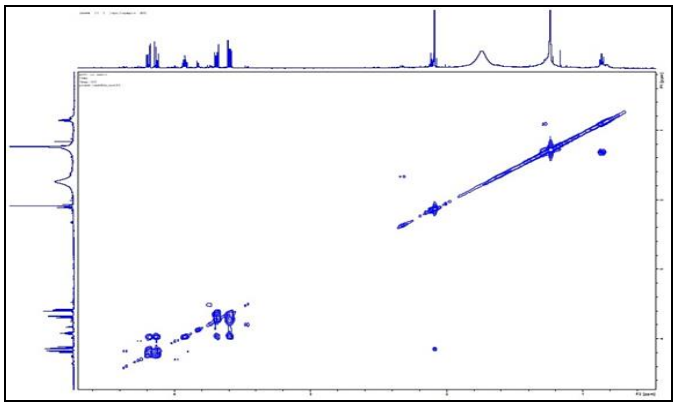

Fig 4: COSY correlation of ADT1

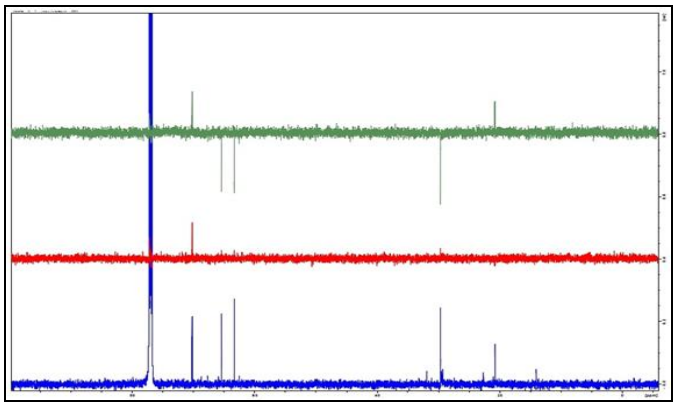

Fig 5: DEPT NMR of ADT1

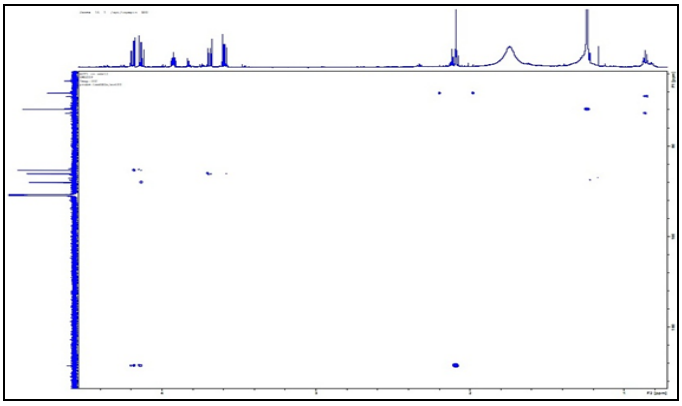

Fig 6: Hmbc Spectrum of Adt1

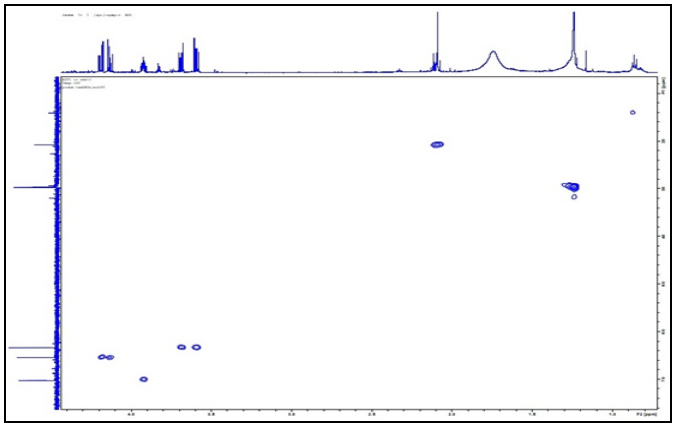

Fig 7: Hsqc Spectrum of Adt1

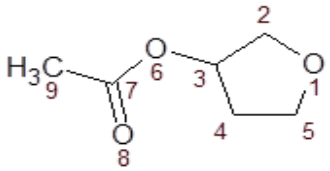

Fig 8: ADT1: Oxolan-3-yl acetate ($C_6H_{10}O_3$, 130.14 g/mol)

Table 5: Sensitivity Test of Compound ADT1

Test organism	Zone of Inhibition (mm)
MRSA	28
VRE	25
S. aureus	29
S. pyogenes	24
S. pneumoniae	0
E. coli	28
N. gonorrhoae	0
S. typhi	0
S. dysenteriae	0
C. albicans	25
C. krusei	22

5. Discussion

5.1 Antimicrobial Screening

Table 1 shows the results of zone of inhibition (ZOI) of the growth of microorganisms against the extracts. It was observed that, there was the highest zone of inhibition for *S. pneumonia* (28mm), followed by *MRSA* (27mm), and the least inhibition was observed on *Candida albicans* (17mm).

The results of the Minimum Inhibition Concentration (MIC) of the extracts are shown in Table 2. Among the extracts screened for antimicrobial activity, the Ethyl acetate extract showed the highest activity against all the microorganisms tested followed by the methanolic extract while the least activity was observed with the hexane extract. The lowest MIC (7.5 mg/mL) and highest MIC (30 mg/mL) values were obtained for the extracts. The methanol, ethyl acetate and n-hexane extracts showed significant activities against *S. typhi*, the bacteria responsible for salmonellosis and typhoid fever. The sensitivity of *E. coli* and *S. aureus* to all the extracts implies that the chemical compound in the extracts could be used to develop drugs to treat related diseases. The use of the plant for the treatment of typhoid fever, diarrhoea and stomach pains is justified since these bacteria may be responsible for these illnesses (Ramanathan *et al*, 2013) [10].

Table 3 shows the result of Minimum Bactericidal Concentration/ Minimum Fungicidal Concentration (MBC/MFC) of the extracts. The low MICs and MBCs/MFCs observed for ethyl acetate extract is an indicator that it may be used as an alternative treatment to orthodox antibiotics in the treatment of disease due to the isolates (Mbata and Saikia, 2007) [7].

5.2 ^{1}H NMR Spectrum of ADT1

The results of the ^{1}H-NMR spectrum of ADT1 shows an oxymethine (H-3) proton which appeared as a multiplet (m) at δ 3.9. Two methylene protons H-2and H-4 appeared downfield as a doublet (dd) at δ 3.6, δ 3.7 respectively, while the upfield singlet(s) at δ 1.25 ppm was assigned to H-5. The methyl protons (H-9) appeared as (s) at δ 2.2 ppm. These assignments were in good agreement with reported values (Banfi *et al*. 2008) [1].

5.3 ^{13}C NMR Spectrum of ADT1

The ^{13}C NMR in combination with the DEPT and HSQC spectra of compound ADT1 has shown recognizable methylene ($-CH_2$) carbon signals at 29.68 ppm, 63.34 ppm and 65.42 ppm which were assigned to C4,C5 and C2 respectively. The signal at 70.22 ppm is due to oxymethine carbon (C3), while the signal at 22.7 ppm is typical of methyl carbon at C9. These signals are characteristics of tetrahydrofurany l esters (Steinbeck *et al.* 2003) [11].

The DEPT experiment spectrum revealed the presence of three methylene carbons at C-4, C-5 and C-2, one oxymethine carbon at C-3, and a methyl carbon at C-9 respectively. The de-shielded signal at (δ_C 70.22) was due to C-3 which is attached to oxygen (Steinbeck *et al.* 2003) [11]. Based on the analysis above, the structure of ADT1 was determined as oxolan-3-yl acetate. The physical and spectral data of ADT1 were in good agreement with those reported in literature for tetrahydrofurany l acetate (Steinbeck *et al*. 2003) [11].

The compound isolated from the ethyl acetate extract of the aerial part of *Vernonioa pauciflora* was a furanyl ester. Esters are among the most widely used class of drugs and their role in therapy of inflammatory, dermatological and oncological diseases has been well described (Rabe *et al.*, 2002) [9].

The ability of the ADT1 to inhibit the growth of several bacterial and fungal species is an indication of the broad spectrum anti-microbial potential of *Vernonia pauciflora* which makes the plant good source for antibiotic and antifungal drugs. Its (oxolan-3-yl acetate) sensitivity against *MRSA, VRE, S. aureus S. pyogenes* indicates that the compound can be further developed for fight against the microorganisms and use of the plant in the treatment of boils, genital infections and skin rashes is justified. The sensitivity of *E. coli, and C. albican* and *C. krusei* to the isolated compound implies that the compound is a potential source of antifever, antidiarrhoea, antinausea and antifatigue and antifungal drugs.

6. Conclusion

The compound oxolan-3-yl acetate was isolated from ethyl acetate fraction of *Vernonia pauciflora.* It was the most potent against *MRSA* and *E. coli (MIC 12.5µg/ml* and MBC

25*μg/ml).* The data obtained from the study of the *V. pauciflora* in the treatment of diseases such as diarrhea, urinary tract infection and skin infection by ethnomedical practitioners have been justified.

We wish to recommend that the test compound be further evaluated to confirm its in vivo activity and toxicity on the host and the mechanism(s) through which the compound exert therapeutic effects should be clearly elucidated.

7. Acknowledgement

Thanks to God Almighty for giving me the wisdom, knowledge and strength to bring this work to completion. My profound gratitude goes to my supervisors; Dr. M. S. Sallau and Dr. H. Ibrahim for their patience, understanding encouragement and support in making this work a success. I am also grateful to Dr. J. D. Habila for providing us with a collaborating laboratory for NMR analysis of the isolate at the University of KwaZulu-Natal, Durban, South Africa.

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